Characterization of molecular events in a series of bladder urothelial carcinoma cell lines with progressive resistance to arsenic trioxide

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Our previous studies have shown that arsenic trioxide (As_2O_3), a novel anti-cancer agent, may be active against urothelial carcinomas. A series of bladder urothelial carcinoma cells with progressive As_2O_3 resistance were established and studied to reveal molecular events in relation to the mechanisms of resistance to As_2O_3. A sensitive parental line (NTUB1) and three As_2O_3-resistant sublines (NTUB1/As) were used with their IC_{50}s being 0.9, 1.2, 2.5 and 4.9 μM, respectively. Cellular resistance to As_2O_3 was associated with a lowered proliferation profile (increased p53 and $p21^{Waf1/Cip1}$ and decreased c-Myc levels) and a greater resistance to apoptosis (elevated Bcl-2 levels). Cells with a stronger resistance had higher expressions of superoxide dismutase (Cu/Zn) and hMSH2 (but not hMLH1). GSH contents were up-regulated in resistant cells in a dose-dependent manner. The DNA-binding activities of NF-κB and AP-1 were down-regulated in resistant cells in a dose-dependent manner. Profound molecular alterations occur during the acquisition of secondary As_2O_3 resistance. Our *in vitro* cellular model may help to reveal resistance mechanisms to As_2O_3 in bladder urothelial carcinoma cells. *Anti-Cancer Drugs* 15:779–785 © 2004 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2004, **15**:779–785

Keywords: arsenicals, DNA repair, drug resistance, p53, reactive oxygen species

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Sponsorship: This work was supported by grants NSC 90-2314-B-002-215, 91-3112-B-002-010 and 92-3112-B-002-024 from the National Science Council, Executive Yuan, Taiwan, ROC.

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Received 30 March 2004 **Revised form accepted** 9 June 2004

Introduction

Although arsenic compounds are known as poisons, they have also been used medicinally for centuries. In 1970s, Thomas Fowler developed a solution (known as Fowler's solution) of potassium arsenite for the treatment of a variety of diseases including asthma, pernicious anemia, Hodgkin's disease, eczema, pemphigus and psoriasis. In 1910, Paul Ehrlich, a Nobel laureate and a pioneer of chemotherapy, introduced salvarsan, an organic arsenical that could cure syphilis and is still used today to treat trypanosomiasis.

Recently, arsenic compounds, such as arsenic trioxide (As_2O_3) and arsenic disulfide, have been used to treat acute promyelocytic leukemia (APL) [1]. The mechanisms of action were shown to be associated with the induction of apoptosis and differentiation [2]. *In vitro* studies revealed that clinically achievable concentrations of As_2O_3 could trigger apoptosis of leukemia [3] and lymphoma [4] cells as well as some solid tumor cells, including those from esophageal cancer [5], prostate cancer [6] and ovarian cancer, etc [6]. This suggests that As_2O_3 may be active against a wide variety of human tumors.

Cell line models that are selected to be secondarily resistant to anti-tumor agents can provide valuable information as to how cells survive toxic environments. Although As_2O_3 has been used in preclinical or clinical settings to treat human cancers, the mechanisms of chemoresistance are not completely understood. Most of the knowledge about arsenic resistance was obtained from a non-cancer models, including those of trypanosoma [7], Chinese hamster ovary cells [8] and human fibroblast cells [9]. The mechanisms of arsenic resistance in human cancer cells have been much less explored. Although it has been suggested that human cells relatively lack the inducible tolerance to arsenite seen in hamster cells [8], we have succeeded in establishing a series of bladder urothelial carcinoma cells that harbor progressive resistance to As_2O_3. These cells are, to our knowledge, the first series of urothelial carcinoma cells that have been selected for resistance to As_2O_3. This is also the first report characterizing the molecular alterations in a series of urothelial carcinoma cells that harbored progressive resistance to As_2O_3. Molecular alterations occurring in these cells may exhibit a dose–response relationship depending on the intensity of arsenic resistance. These cells therefore may represent an

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excellent cellular model for revealing the arsenic-mediated molecular events and its chemoresistance mechanisms in human cancer cells.

Materials and methods

Cell lines and chemicals

NTUB1 is an immortalized human urothelial carcinoma cell line raised from a high-grade bladder cancer [10]. A series of As_2O_3-resistant sublines were developed by chronically exposing NTUB1 to progressively increased concentrations of As_2O_3 (Sigma, St Louis, MO). Three sublines that can survive at 0.1, 0.2 and 0.4 μM As_2O_3 were used in the study, and were designated as NTUB1/As(0.1), NTUB1/As(0.2) and NTUB1/As(0.4), respectively. All cells were cultured in an RPMI 1640 medium (Gibco/BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (Gibco/BRL) at 37°C in humidified air with 5% CO_2.

Chemosensitivity assay

Cellular chemosensitivity to As_2O_3 was studied using a modified MTT (Sigma) assay to determine cell viability *in vitro* [11]. Cells (NTUB1: 4000, NTBU1/As(0.1): 5000, NTUB1/As(0.2): 7000 and NTUB1/P(0.4): 9000 cells/well, respectively) were cultured in 96-well microplates and incubated with graded concentrations of As_2O_3 at 37°C for 72 h. Plated cell numbers were titrated to keep control cells growing in the exponential phase throughout the 72-h incubation period. After exposure for 72 h, 50 μl of MTT (2 mg/ml in RPMI medium) was added to each well and allowed to react for 2.5 h. The blue formazan crystals that formed were pelleted to well bottoms by centrifugation, separated from the supernatant and dissolved in 150 μl of dimethylsulfoxide. The proportion of surviving cells was determined by the absorbance spectrometry at 492 nm using an MRX-2 microplate reader (Dynex, Chantilly, VA). Three independent experiments with triplicate data were performed. The As_2O_3 IC_{50}s of these cells were calculated by the median-effect equation [12] and presented as mean ± SEM.

Cellular growth rate curve

The cellular growth rate curve was also studied using the MTT assay, similar to the chemosensitivity assay using a modified MTT (Sigma) assay to determine cellular growth *in vitro*. The four kinds of cells (5000 cells/well) were cultured in 96-well microplates and incubated with various concentrations of As_2O_3 (0, 0.1, 0.2 and 0.4 μM, respectively) at 37°C for 0, 24, 48, 72 and 96 h, respectively. After exposure for various time course, the following steps were previously described as MTT assay. Three independent experiments with triplicate data were performed and data presented as mean ± SEM.

Western blotting

To dissect the molecular alterations occurring in the series of cells, we studied the protein levels of the three cell cycle regulators (p53, $p21^{Waf1/Cip1}$ and c-Myc), the anti-apoptosis factor (Bcl-2), the reactive oxygen species (ROS) scavenger [superoxide dismutase (Cu/Zn)], and the two DNA mismatch repair enzymes (hMSH2 and hMLH1) by using Western blotting analysis. Cellular protein collected and concentration determinations were carried out as described in a previous study [13]. Cells scraped from a 100-mm Petri dish were resuspended in 100 μl of gold lysis buffer (10% glycerol, 1% Triton X-100, 137 mM NaCl, 10 mM NaF, 1 mM EGTA, 5 mM EDTA, 1 mM sodium pyrophosphate, 20 mM Tris–HCl, pH 7.9, 100 μM β-glycerophosphate, 1 mM sodium orthovanadate, 0.1% SDS, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin) and put on ice for 30 min. The lysate was then centrifuged at 18 000 *g* for 30 min at 4°C to collect the supernatant for protein concentration determination with the BCA Protein Assay Reagent (Pierce Life Science, Rockford, IL) and for Western blotting analysis. Briefly, protein extracts (50 μg) were separated on 10% SDS–polyacrylamide gels and transferred to microporous PVDF membranes (Boehringer Mannheim, Mannheim, Germany). After blocking with the TBST buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl and 0.1% Tween-20) plus 1% bovine serum albumin, the membranes were incubated with human-specific antibodies at 4°C for 12–18 h against the target proteins. These antibodies were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA; Bcl-2 and p53) and PharMingen [San Diego, CA; $p21^{Waf1/Cip1}$, superoxide dismutase (Cu/Zn) and c-Myc] as monoclonal antibodies, and from Santa Cruz Biotechnology (hMSH2 and hMLH1) as polyclonal antibodies. The membranes were washed 3 times with the TBST buffer (20 min each) and incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. After 3 washes with the TBST buffer, these proteins were detected by Western blotting Luminol reagent (Santa Cruz Biotechnology).

Electrophoretic mobility shift assay (EMSA)

The nuclear DNA-binding activities of the two transcription factors, NF-κB and AP-1, were studied by using EMSA. Nuclear proteins were extracted based on our previously described methods [14] by adding 500 μl of cold hypotonic buffer [20 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.5 mM PMSF] to the cell pellets that were put on ice for 15 min. The mixture was then added to 30 μl of 10% NP-40 and vortexed for 10 s. The mixture was centrifuged at 3000 *g* for 5 min at 4°C to collect the pellets that were then resuspended in 50 μl of cold hypertonic buffer (20 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 400 mM NaCl) and agitated vigorously at 4°C for 15 min. Cell debris was removed by centrifugation at 3000 *g* at 4°C for 5 min. Binding reactions were performed while on ice for 20 min with 5 μg nuclear protein in 15 μl of 10 mM Tris–HCl

(pH 7.5), 50 mM NaCl, 1 mM EDTA, 10% glycerol, 2 μg poly(dI–dC), 1 mM DTT, 1 mM PMSF and 30 000 c.p.m. ^{32}P-labeled oligonucleotides. DNA–protein complexes were separated from unbound DNA probes on native 6% polyacrylamide gels. Gels were vacuum dried and exposed to Kodak films at –20°C for 16–48 h. The sequences of the NF-κB and AP-1 oligonucleotides were as follows: NF-κB: 5′-AGTTGAGGGGACTTTCC-CAGG-3′; AP-1: 5′-TGACTA-3′.

Cellular glutathione (GSH) content

Cells (3×10^6) were resuspended in 0.5 ml of 5% metaphosphoric acid (Sigma) and put on ice for 20 min. Cells were then lysed by 3 cycles of freeze–thaw. After centrifugation at 4°C for 20 min, the supernatant was collected. Cellular GSH content was determined using the GSH-400 kit (Oxis International, Portland, OR) according to the instructions of the manufacturer [13]. Briefly, 50 μl of supernatant was incubated with 0.4 ml of the reaction buffer (200 mM potassium phosphate, 0.2 mM diethylene-triamine pentaacetic acid and 0.025% lubrol, pH 7.8). Then, 25 μl of chromogenic reagent in 0.2 N HCl and 25 μl of 30% NaOH were sequentially added and mixed thoroughly. The mixture was reacted in the dark at room temperature for at least 10 min. The colorimetric density of the target chromogen was determined by a spectrophotometer (DU640i; Beckman, Fullerton, CA) at 400 nm. Three separate experiments with triplicate data were performed.

Statistical methods

All symmetrical numeric data were presented as mean ± SEM. To determine if higher cellular GSH contents were associated with the stronger As_2O_3 resistance, linear regression analysis was used with the IC_{50}s of the four cell lines, with GSH contents being the independent and dependent variables, respectively. The regression analysis was carried out using SAS software for an IBM-compatible PC (version 8.0, 2000).

Results

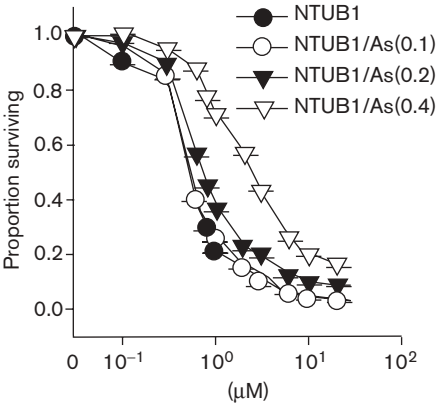
IC_{50}s of the series of cell lines

The As_2O_3 IC_{50}s of NTUB1, NTUB1/As(0.1), NTUB1/As(0.2) and NTUB1/As(0.4) were 0.9, 1.2, 2.5 and 4.9 μM, respectively. The IC_{50} of the most resistant subline, NTUB1/As(0.4), is 5.4-fold higher than that of NTUB1 (Fig. 1).

Regulators of cell proliferation and cellular growth rate

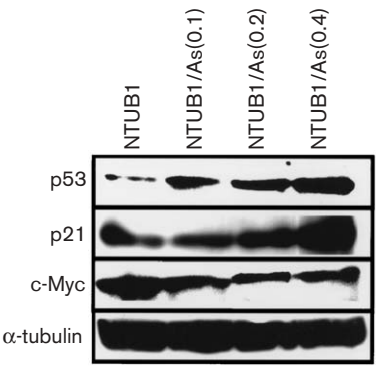
As shown in Fig. 2, the p53 and p21$^{Waf1/Cip1}$ protein levels were positively correlated with the As_2O_3 resistance intensity, with an evident dose–response relationship. In sharp contrast, c-Myc levels, the transcription factor that enhances cell proliferation, were down-regulated in the three resistant cells. In accordance with this, the *in vitro* growth rates of the As_2O_3-resistant cells were consistently found to be slower than the parental NTUB1 cells (Fig. 3). It appeared that the proliferation activities of A_2O_3-resistant cells were significantly slowed down compared to the parental NTUB1 cell.

Fig. 1



Chemosensitivity assay. Chemosensitivity curves of the four cells to As_2O_3 determined by the MTT assay. The As_2O_3 IC_{50}s of NTUB1, NTUB1/As(0.1), NTUB1/As(0.2) and NTUB1/As(0.4) were 0.9, 1.2, 2.5 and 4.9 μM, respectively, with NTUB1/As(0.4) being 5.4-fold higher than NTUB1. All symmetrical numeric data are presented as mean ± SEM.

Fig. 2

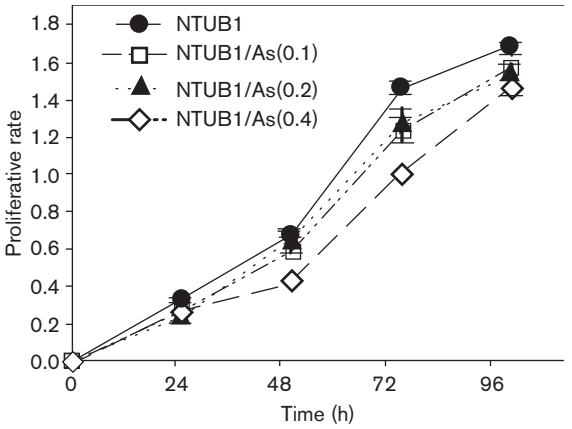


Cell proliferation profiles of the series of As_2O_3-resistant urothelial carcinoma cell lines determined by the Western blotting analysis. The greater the resistance to As_2O_3, the lower the proliferation profile as shown by higher p53 and p21 expressions and lower c-Myc levels. α-Tubulin served as the internal control.

Induction of apoptosis-resistant protein

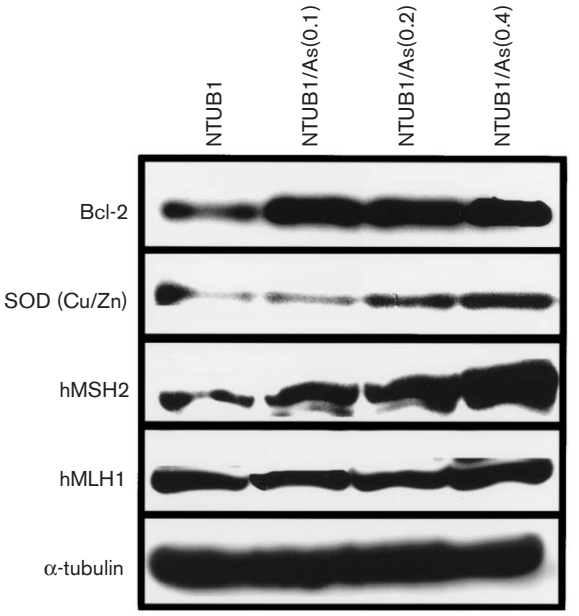
The protein levels of Bcl-2 were evidently up-regulated in A_2O_3-resistant cells (Fig. 4). However, there was no dose–response relationship between the expression levels and the resistance capacity. This suggests that the resistant cells were more capable of surviving in an unfavorable environment that would normally kill sensitive cells.

Fig. 3



Cellular growth rates. The parental and three As_2O_3-resistant urothelial carcinoma cells were maintained at 0, 0.1, 0.2 and 0.4 μM As_2O_3, respectively. Cellular growth curves of the four cells were determined by the MTT assay. All symmetrical numeric data are presented as mean ± SEM.

Fig. 4



Western blotting of Bcl-2, superoxide dismutase (Cu/Zn) and DNA repair enzymes (hMSH2 and hMLH1) in the series of As_2O_3-resistant urothelial carcinoma cells. Cells with greater resistance had higher levels of Bcl-2, superoxide dismutase (Cu/Zn) and hMSH2, but not hMLH1. α-Tubulin served as the internal control.

Induction of ROS scavenger and DNA repair enzymes

The protein levels of superoxide dismutase (Cu/Zn) were progressively increased in the three resistant cell lines (Fig. 4). Similarly, the DNA mismatch repair enzyme, hMSH2 (but not hMLH1), was up-regulated in the resistant cells in a dose-dependent manner (Fig. 4). These data imply that cellular resistance to A_2O_3 may involve the up-regulation of cells capability to survive oxidative stress and to fix mismatched DNA structures.

GSH contents

The GSH contents of NTUB1, NTUB1/As(0.1), NTUB1/As(0.2) and NTUB1/As(0.4) were 189.3 ± 7.4, 225.4 ± 9.7, 233.2 ± 9.2, and 260.4 ± 10 μM/μg protein, respectively. The stronger cellular As_2O_3 resistance, the higher GSH contents ($p = 0.0002$, $R^2 = 0.48$). This suggests that cellular resistance to As_2O_3 is associated with increased cellular GSH levels.

Nuclear NF-κB and AP-1 DNA-binding activity

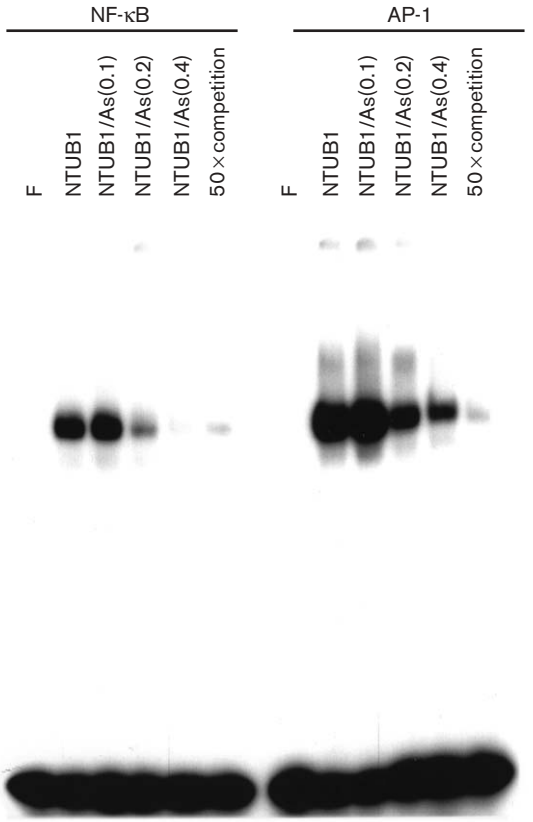
The constitutional nuclear DNA binding activity of both NF-κB and AP-1 were significantly reduced in resistant cells in a dose-dependent manner (Fig. 5). Although the binding activity of NF-κB in NTUB1/As(0.1) remained unchanged as compared to the control NTUB1 cells, it was barely detectable in NTUB1/As(0.2) and nearly absent in NTUB1/As(0.4). Control competition experiments using a 50-fold excess of unlabeled (cold) oligonucleotides can significantly block the binding of the two transcription factors to the respective hot oligonucleotides, which indicates that the binding reactions were specific.

Discussion

This is the first report characterizing the molecular alterations in a series of urothelial carcinoma cells that harbored progressive resistance to a novel cytotoxic agent, As_2O_3. Molecular events occurring in a series of cells may provide valuable information since the dose–response relationship between drug resistance and existing molecular events further substantiates the association between them. In this study, we have shown that cellular resistance to As_2O_3 in urothelial carcinoma cells is associated with the down-regulation of cell proliferation activities, and up-regulation of a wide variety of cellular detoxification machineries that include the cellular GSH, DNA repair and ROS scavenging systems. Dose-dependent suppression of NF-κB and AP-1 activation was also demonstrated in resistant cells.

p53 and $p21^{Waf1/Cip1}$ are known to function as a checkpoint in cell cycle progression. They usually halt progression and drive cells toward apoptosis in cases of unrecoverable DNA damages [15]. In contrast, the c-Myc oncoprotein is associated with uncontrolled cell division [16]. In our results, the p53 and $p21^{Waf1/Cip1}$ proteins were up-regulated, and c-Myc was down-regulated in a dose-dependent manner, which together obviously function to put a brake on cell cycle progression and slow down cell proliferation. In accordance with this, the growth rates of the As_2O_3-resistant cells were consistently found to be

Fig. 5



Reduction of NF-κB and AP-1 DNA-binding activity in As_2O_3-resistant urothelial carcinoma cells as determined by EMSAs. The higher the resistance to A_2O_3, the lower the constitutional activity of both NF-κB and AP-1. Control competition experiments using a 50-fold excess of unlabeled (cold) oligonucleotides (× 50 competition) significantly blocked the binding of the two nuclear factors to the respective hot oligonucleotides, indicating specific binding.

slower than the parental NTUB1 cells. The reason why resistant cells had a slower proliferation rate than sensitive cells is not clearly understood. It seems that cells that have to spend the majority of energy to survive in an undesirable toxic environment would have less spare energy for growth and proliferation. In a previous report, we have shown that the expression of *mdr-1*, a membrane-bound energy-dependent efflux pump that is responsible for the phenotype of multiple drug resistance, was seen in 100% of normal urothelial mucosal samples, 70% of clinical urothelial cell tumors, but only 20% of the 10 urothelial carcinoma cell lines [17]. Cultured cancer cells that usually have a higher proliferation rate than tumors and normal tissues may somehow evolve to inactivate various energy-consuming mechanisms, permitting rapid cell growth and proliferation. Conversely, resistant cells that have developed various energy-consuming resistance machineries have to slow down cell growth and proliferation. This may at least in part explain why the As_2O_3 cells had a lowered proliferation profile.

It was shown that As_2O_3 at low doses selectively inhibits growth and induces apoptosis of APL cell line NB4 [2]. The mechanisms of As_2O_3-mediated apoptosis were shown to involve the down-regulation of the Bcl-2 protein [2] and the activation of the ROS-related caspase-3 pathway [18]. The protein levels of Bcl-2 were up-regulated in the three resistant cells, which suggests that cancer cells tend to resist apoptotic stresses by developing a mechanism to survive toxic and lethal environments. In addition, the activated Bcl-2 pathway and its related survival mechanism may help escape from lethal attacks of other cytotoxic agents and thus produce a cross-resistance to agents other than As_2O_3. In a previous report, we first demonstrated that cross-resistance between As_2O_3 and cisplatin may occur in urothelial carcinoma cells [19]. The cross-resistance may arise from the sharing of common resistance mechanisms, such as elevated GSH contents, ROS scavenging capacity and DNA repair capacity [13,20]. This phenomenon should be considered in advance when designing second-line protocols for urothelial carcinomas.

In human cells, five DNA mismatch repair proteins, i.e. hMSH2, hMSH3, hMSH6, hMLH1 and hPMS, have been identified as responsible for the identification and correction of DNA replication errors [21]. Loss of their functions may result in the accumulation of DNA replication errors and mutant phenotypes. In contrast, enhancement of these repair functions may help cells to survive otherwise lethal DNA damage caused by a variety of cytotoxic agents. Previous studies have shown that arsenic compounds may inhibit DNA repair activity and result in DNA damage. Mismatch errors have been identified as a major form of arsenic-mediated DNA insults [22]. In addition to mismatched nucleotides, these repair proteins have been found to recognize specific types of DNA lesions. For example, the complex of hMSH2–hMSH6 may directly recognize cisplatin-induced DNA adducts and facilitate the transcription-coupled DNA repair processes [23]. In fact, it has been shown that As_2O_3-generated ROS could attack DNA and result in the formation of DNA adducts during the carcinogenesis of human skin [24]. In our results, the hMSH2 protein was progressively up-regulated in the three resistant cells, which suggests that As_2O_3-resistant cells manage to up-regulate the DNA mismatch repair machinery which then protects cells from As_2O_3-mediated DNA damage and cell death. These findings imply that hMSH2 is involved in the recognition of As_2O_3-generated DNA adducts and activation of the transcription-coupled repair mechanism in urothelial carcinoma, which is similar to the involvement of hMSH2 in the recognition of cisplatin-mediated DNA adducts.

A previous study showed that the mechanism of self-tolerance to low-dose arsenite in liver epithelial cells was based primarily on reduced cellular disposition of the

metalloid and was not accounted for by changes in levels of GSH or the metalloid [25]. However, we demonstrated that the GSH contents were significantly elevated in As_2O_3-resistant cells in a dose-dependent manner. GSH is the major cellular antioxidant that functions to scavenge free radicals and detoxify cytotoxic substances. In cells of low GSH content, arsenic binds to sulfhydryl group-containing compounds such as GSH. The capacity to eliminate ROS is then decreased, which results in increased oxidative stress and cell death [18]. Except for GSH content, we also showed that the ROS scavenger, superoxide dismutase (Cu/Zn) plays an important role in As_2O_3 resistance. Cells with a higher As_2O_3 resistance were found to have higher levels of superoxide dismutase (Cu/Zn). The above data suggest that the cytotoxic effects and the resistance mechanisms of As_2O_3 are closely related to their cellular abilities to resist oxidative stress. Our recent data have also shown that buthionine sulfoximine may effectively reduce the cellular capacity of anti-oxidation, and restore the sensitivity to both cisplatin and As_2O_3 [19,26].

The constitutional nuclear DNA-binding activities of NF-κB and AP-1 were significantly reduced in resistant cells. Although NF-κB has been implicated in the control of apoptosis and oncogenesis [27], conflicting data exist in relation to the role of NF-κB in programmed cell death. A number of recent studies have shown that NF-κB is an important pro-apoptotic factor for some specific agent-mediated apoptosis [28]. In the progenitor B lymphocytes, inhibition of NF-κB activation delays cytokine withdrawal-induced cell death [29]. However, it was also demonstrated that NF-κB has an anti-apoptotic function in response to tumor necrosis factor-α-induced apoptosis [30]. Therefore, the actual role of NF-κB, as being an anti- or pro-apoptotic protein, may depend on the different responding tissue systems and distinct triggering agents. In our cellular model, it appears that NF-κB is pro-apoptotic so that resistant cells managed to suppress their nuclear DNA-binding activity so as to survive in an unfavorable situation. Interestingly, expression of Fas ligand was found to be regulated by NF-κB and AP-1 [28], which may also suggest that down-regulation of both NF-κB and AP-1 is related to the anti-apoptotic capacity of A_2O_3-resistant cells.

The mechanisms of As_2O_3 resistance in urothelial carcinoma cells involve multiple pathways. Profound molecular events occur during the acquisition of As_2O_3 resistance, including lowered proliferation activity, increased resistance to apoptosis, up-regulated detoxification mechanisms against oxidative stress and elevated DNA mismatch repair functions. Our cellular model may help to reveal molecular events in relation to secondary As_2O_3 resistance in human urothelial carcinomas.

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